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(54) Title: GnRH ANALOGS FOR DESTROYING GONADOTROPHS

(57) Abstract

Certain toxic compounds (T) such as, for example, compounds based upon diphtheria toxin, ricin toxin, pseudomonas exotoxin, α-amanitin, pokeweed antiviral protein (PAP), ribosome inhibiting proteins, especially the ribosome inhibiting proteins of barley, wheat, corn, rye, gelonin and abrin, as well as certain cytotoxic chemicals such as, for example, melphalan and daunorubicin can be conjugated to certain analogs of gonadotropin-releasing hormone to form a class of compounds which, when injected into an animal, destroy the gonadotrophs of the animal's anterior pituitary gland. Hence such compounds may be used to sterilize such animals and/or to treat certain sex hormone related diseases.

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GnRH ANALOGS FOR DESTROYING GONADOTROPHS

FIELD OF THE INVENTION

The present invention generally relates to methods for sterilizing animals and to methods for medically
5 treating certain sex hormone related diseases such as, for example, cancer of the breast or prostate. More particularly, this invention relates to sterilization and medical treatment by means of chemical attack upon the pituitary gland.

10 ## BACKGROUND OF THE INVENTION

Considerable interest exists with respect to the subject of sterilization of animals. This is especially true of those concerned with veterinary medicine and animal husbandry, particularly as they
15 relate to the subject of sterilization of domestic animals such as dogs, cats, cattle, sheep, horses, pigs, and the like. Various methods have been developed over the years to accomplish sterilization. For example, with respect to male cattle, the most
20 widely used procedure for eliminating problems of sexual or aggressive behavior is sterilization through surgical castration. This is done in various ways, e.g., crushing the spermatic cord, retaining the testes in the inguinal ring, or use of a rubber band,
25 placed around the neck of the scrotum, to cause sloughing off of the scrotum and testes. However most

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of these "m chanical" castration methods have proven
to be undesirable in one resp ct or another; for
example they (1) are traumatic, (2) introduce the
danger of anesthesia, (3) are apt to produce
5   infection, and (4) require trained personnel.
Moreover, all such mechanical castration methods
result in complete abolition of the testes and this of
course implies complete removal of the anabolic
effects of any steroids which are produced by the
10   testes and which act as stimuli to growth and protein
deposition.

These drawbacks have caused consideration of
various alternative sterilization techniques such as
the use of chemical sterilization agents. However,
15   the use of chemical sterilization agents has its own
set of advantages and disadvantages. On the positive
side, chemical sterilization eliminates the stress and
danger associated with mechanical castration.
Chemical sterilization also has the added advantage of
20   allowing for retention of certain anabolic effects
resulting from a continued presence of low levels of
circulating testosterone. This is especially valuable
in the case of animals raised for human consumption
since circulating testosterone promotes growth,
25   efficiency of feed conversion and protein deposition.
Unfortunately, there are several disadvantages
associated with chemical sterilization. For example
chemical sterilization is often temporary rather than
permanent; it also sometimes produces extremely
30   severe, and even fatal, side effects.

Many of these chemical sterilization methods have
been aimed at regulation of luteinizing hormone
produced at various stages of an animal's sexual
development. For example, with respect to cattle it
35   has been established that in the case of the infantile

calf, luteinizing hormone is rarely discharged and
testicular production of androgens is at low levels.
On the other hand, in a prepubertal calf, or an adult
bull, discharges of luteinizing hormone from the

5   anterior pituitary occur more frequently and the
testes produce considerably larger amounts of
testosterone and other steroids. It is thought that
these conditions result from the following factors:
(1) decreases in the concentration of estradiol

10   receptors in the hypothalamus, (2) concomitant
increases in the concentration of estradiol receptors
in the anterior pituitary, and (3) increases the
number of gonadotropin-releasing hormone (GnRH)
receptors in the anterior pituitary. This increase in

15   GnRH receptors is generally regarded as a prerequisite
for an animal to pass from the infantile stage to the
prepubertal and mature stages of endocrine
development. Hence, based upon these understandings
of the hypothalamic-pituitary-testicular axis, several

20   chemical methods have been proposed to modify given
animals, e.g., a bull calf, in such a way that it
never enters puberty, but still receives stimuli for
growth and protein deposition through the anabolic
effects of steroids produced by modified testes. In

25   any event, most of the chemicals proposed for such
sterilization purposes are hormones or hormone
analogs. For example U.S. Patent 4,444,759 teaches
the use of a class of peptides analogous to GnRH
(i.e., gonadotropin-releasing hormone, and

30   particularly luteinizing hormone-releasing hormone)
capable of inhibiting release of gonadotropins by the
pituitary gland and thereby inhibiting release of the
steroidal hormones, estradiol, progesterone and
testosterone. It should also be noted that the terms

35   "GnRH" (gonadotropin-releasing hormone) and "LHRH"

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(luteinizing hormone-releasing hormone) ar sometimes
used interchangably in the literature.   For the
purposes of describing the prior art both terms may be
employed; however, for the purposes conveying the
5   teachings of our patent disclosure, applicants prefer
the term GnRH and will use it in describing their
compounds.

Be that as it may, some prior art chemical
sterilization procedures are specifically adopted to
10   alter luteinizing hormone secretion before the animal
has attained the age of puberty.   This is not
surprising since the role of luteinizing hormone in
sexual maturation is well known.   Luteinizing hormone
is a gonadotropic hormone found in the anterior lobe
15   of the pituitary gland and, in male animals, it is
known to stimulate the interstitial cells of the
testes to secrete testosterone (see generally, The
Merck Index, 8th edition, p. 560 (1968), Encyclopedia
of Chemical Technology, Vol. 7, pp. 487-488 (1951)).
20   One approach has been to use certain chemicals to
produce antibodies in an animal which exhibit cross-
reactivity with the gonadotropins produced by the
animal's pituitary.   It is generally thought that with
such early antigenic stimulation, formation of
25   antibodies is more continuously stimulated by the
release of endogenous hormones and that early
immunization with such luteinizing hormone deters the
maturation of the gonads and adnexal glands.   This, in
turn, is thought to inhibit spermatogenesis at the
30   spermatogonial level.   However, early immunization of
this kind also tends to make the interstitial tissues
fibroblastic.   It has also been found that such early
stimulation of the immunologic system leads to
development of a high titered antiserum to luteinizing
35   hormone which remains at relatively high levels.

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Nonetheless, periodic boosters are often necessary for adult animals sterilized befor puberty in order to maintain high levels of the n utralizing antibodies.

Similarly, luteinizing hormone has been
5   administered to animals after they have attained the age of puberty in order to atrophy their reproductive organs and to cause a decrease in libido (see generally, M. Tallau and K.A. Laurence, Fertility and Sterility, Vol. 22, No. 2, February 1971, pp. 113-118,
10   M.H. Pineda, D.C. Lueker, L.C. Faulkner and M.L. Hopwood, Proceedings of the Society for Experimental Biology and Medicine, Vol. 125, No. 3, July 1967, pp. 665-668, and S.K. Quadri, L.H. Harbers, and H.G. Spies, Proc. Soc. Exp. Biol. Med., Vol. 123, pp. 809-
15   814 (1966). Such treatments also impair spermatogenesis in noncastrated adult male animals by interruption of the spermatogenic cycle.

Other chemical sterilization agents have been specifically designed for use on female animals. For
20   example, it is well known that certain antigens will produce an antiserum against a requisite estrogen. This is accomplished by first making an antigen and then injecting said antigen into an animal for purposes of antiserum production. The animal is then
25   bled to recover the antiserum. Any female animal of the same species as the host animal may then be injected with the antiserum at the proper time prior to ovulation and the injected antiserum will cause temporary sterilization of that animal.

30   Other methods of chemical sterilization have been based upon direct chemical attack upon certain cells of the pituitary itself (as opposed to chemical attacks upon the hormone products of such cells) with a view toward permanently destroying such cells.
35   Again, this approach is suggested by the fact that

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follicle stimulating hormon (FSH) and luteinizing hormone (LH) (sometimes r ferred to as gonadotropins or gonadotropic hormones) are released by the pituitary gland to regulate functioning of the gonads to produce testosterone in the testes and progesterone and estrogen in the ovaries. They also regulate the production and maturation of gametes.

Several chemical agents have been proposed for this purpose. However, it has been found that most chemical agents which are in fact capable of destroying the gonadotrophs of an animal's anterior pituitary gland also tend to produce extremely toxic side effects which can severely weaken, and sometimes kill, the treated animal. Hence, with respect to the general subject of chemical sterilization, it can be said that any chemical capable of producing sterilization without, or with minimal, toxic side effects would be of great value in the fields of animal husbandry, veterinary medicine and wildlife control.

Knowledge of the above noted sex hormone functions also has produced several advances in the field of human medicine. For example, the potential for achieving chemical castration (rather than "surgical" castration) with certain luteinizing hormone-releasing hormone (LHRH) analogs has been reported (see for example, Javadpour, N., Luteinizing Hormone-Releasing Hormone (LHRH) in Disseminated Prostatic Cancer; IM, Vol. 9, No. 11, November 1988). Table I below gives the structure of LHRH and the structure of certain analogs (e.g., Goserelin, Leuprolide, Buserelin and Nafarelin) of LHRH which are capable of temporarily suppressing luteinizing hormone secretion and thereby suppressing the gonads. As a consequence, these LHRH analogs have come to be regarded as a promising new

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class of agents for the treatment of various host-dependent diseases, especially prostatic cancer. In referring to Table I, it first should be noted that LHRH has a decapeptide structure and that substitution of certain amino acids in the sixth and tenth positions of the LHRH produce analogs which render agonists that are up to 100 times more potent than the parent LHRH compound (hence these compounds are often referred to as "superagonists"). Structures of LHRH and the most commonly known LHRH superagonists are listed in Table I.

TABLE I

STRUCTURES OF LHRH AND SOME SUPERAGONISTS
(Superagonists have substitutions at
positions 6 and 10)

LHRH: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Amide
       1   2   3   4   5   6   7. 8   9   10

SUPERAGONISTS:

Name	Subs. at 6	Subs. at 10	Terminator
Goserelin:	D-Ser(tBu)	AzaGly	Amide
Leuprolide:	D-Leu	des-Gly	Ethylamide
Buserelin:	D-Ser(tBu)	des-Gly	Ethylamide
Nafarelin:	D-2-NaphthylAla	None	Amide

While these compounds represent the most promising means for palliative therapy because of their relative lack of side effects, they remain expensive and must be administered repeatedly. Even the newest formulations utilizing polymer encapsulated drug or other depot forms will require at least monthly administration.

Depot forms are presently in development, but they

too are likely to be qually expensive and also requir monthly administration. In response t these drawbacks, Applicants have develop d a class of compounds which is capable of producing safe,

5   inexpensive, chemical castration as an alternative to surgical castration. Such drugs also greatly simplify therapy of the generally elderly patients with prostate cancer, and could eliminate the need for surgical castration (still preferred by many

10 urologists) as well as provide a medical alternative to oophorectomy in females with advanced breast cancer. Moreover, as a model system, the ability to eliminate pituitary gonadotrophs in vivo, which are regulated by GnRH receptors in response to ligand

15 stimulation in a predictable fashion, is a highly appealing first step toward the more complex use of toxin conjugates to antibodies to eliminate tumor targets. Hence, use of applicants' compounds generally will fall into two major areas of use. The

20 first is sterilization of mammals of all types; the second is chemical castration of mammals in general, and human beings in particular, for purposes of treating breast or prostate cancer by ablating those pituitary cells, namely gonadotrophs responsible for

25 LH secretion.

SUMMARY OF THE INVENTION

The present invention provides a group f toxin compounds, agents and processes for effectively sterilizing animals (e.g., mammals such as cattle, sheep and humans, as well as fowl and fish) and/or for treating certain sex hormone related diseases such as cancer of the prostate and cancer of the breast, especially in humans. These compounds also have utility as agents for aiding in putting weight on (e.g., increasing the rate of metabolic formation of protein) cattle and other meat producing animals. These compounds or agents may also be referred to as "toxic compounds", ("T") or "toxins" for the purposes of this patent disclosure. In any event, the most effective, and hence most preferred of these compounds (toxins) are based upon diphtheria toxin, ricin toxin, pseudomonas exotoxin, shiga toxin, α-amanitin, pokeweed antiviral protein (PAP), ribosome inhibiting proteins (RIP), especially the ribosome inhibiting proteins of barley, wheat, flax, corn, rye, gelonin, abrin, modeccin and certain cytotoxic chemicals such as, for example, melphalan, methotrexate, nitrogen mustard, doxorubicin and daunorubicin. All of these toxins are characterized by their lack of ability, in their own right, to chemically attack the gonadotropin-secreting cells of the anterior pituitary gland.

Applicants have, however, found that these sterilization agents can be effectively delivered to the pituitary gland while chemically conjugated with certain peptide hormone molecules such as certain analogs of gonadotropin-releasing hormone, GnRH. Other peptide hormone molecules (other than gonadotropin-releasing hormone) to which the herein disclosed toxins could be conjugated are human

chorionic gonadotropin, equine chorionic gonadotropin,
luteinizing hormone and follicle-stimulating hormone.
At this point, it should also be noted that for the
purposes of this patent disclosure, the term
5   gonadotropin-releasing hormone will usually be
abbreviated as "GnRH". However, it should also be
noted that for the most part certain hereinafter
described analogs of GnRH are far more effective
carrier peptide hormone molecules for the practice of
10 this invention than the basic or parent GnRH molecule.
In their most generalized sense, these analogs will be
abbreviated as "GnRH-A", with the "A" designating that
the resulting compound is an analog of the basic GnRH
molecule. Similarly, in their most generalized sense,
15 any general toxin compound which is conjugated to the
GnRH-A molecule will be abbreviated by the letter "T"
for toxin. Thus the abbreviation for a generalized
conjugate of the GnRH-A and toxin will be "GnRH-A-T".
In certain hereinafter described preferred cases
20 the concept of an "analog" of the GnRH also will
include those molecules wherein the analog includes a
linkage or coupling moiety specifically intended to
link the GnRH-A group or moiety to the toxic group or
moiety. Most preferably such toxic compounds (T) and
25 their associated carrier peptide molecules are
covalently linked by a linking or coupling agent
selected from the group consisting of N-succinimidyl
3-(2 pyridyldithio) propionate (SPDP), 2-
iminothiolane, thioether, carbodiimide, and epsilon
30 amino caproic acid. In the case of a GnRH-A carrier
peptide molecule, this linking or coupling is
preferably carried out at the 6 position of the parent
GnRH molecule. That is to say that most preferable
production of such "analogs" of the basic GnRH
35 molecule will involve modification of the 6 position

of the GnRH molecule. Amino acid substitutions at th
6 position of the GnRH molecule yield analogs with
high affinity for GnRH receptors and also provide a
means for covalently linking the toxin molecule to the

5   GnRH analog. The most preferred amino acids for this
purpose are lysine, D-lysine, aspartic acid, D-
aspartic acid, glutamic acid, D-glutamic acid,
cysteine, D-cysteine, ornithine, D-ornithine tyrosine,
D-tyrosine, and other amino acids having suitable

10 side-chain functional groups such as, for example,
amino groups, carboxylic groups or sulfhydryl groups.
Similarly the 10 position of the basic GnRH molecule
can be modified to produce other analog variations.
Addition of groups such as Gly-NH_2, ethylamide, or

15 Aza-Gly-NH_2 to the Pro at the 9 position of the basic
GnRH molecule produce preferred analog forms. A
structural depiction of the most preferred members of
the resulting group of compounds of this patent
disclosure is given in TABLE II.

20

TABLE II
(Preferred GnRH-A-T Compounds)

```
pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Z
  1   2   3   4   5   6   7   8   9  10
25                            |
                             X
                             |
                             Y
                             |
30                           T
```
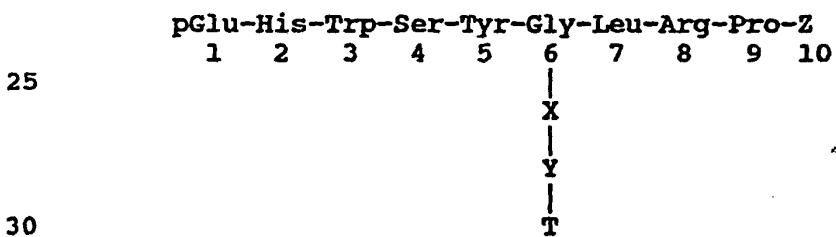
Wherein the pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro
                1   2   3   4   5   6   7   8 9-Gly

10   represents the GnRH group, X is an amino acid
selected from the group consisting of: tyrosine, D-
35 tyrosine, ornithine, D-ornithine, glutamic acid, D-
glutamic acid, aspartic acid, D-aspartic acid,

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cysteine and D-cysteine; Y is a linking agent (wh se
use is optional, but highly preferred) selected from
the group consisting of: carbodiimide, 2-
iminothiolane, SPDP, (N-succinimidyl 3-(2-
5   pyridyldithio) propionate), bis-diazobenzidine and
glutaraldehyde; Z is a chemical group selected from
the group consisting of Gly-NH$_2$, ethylamide, and Aza-
Gly-NH$_2$ and T is a toxin group, capable of chemically
attacking the gonadotrophs of the pituitary gland when
10   conjugated to the carrier peptide molecules described
in this patent disclosure. Such toxins can be
selected from the group consisting of the plant
toxins: ricin, modeccin, abrin, pokeweed anti-viral
protein, α-amanitin, gelonin ribosome inhibiting
15   protein ("RIP"), barley RIP, wheat RIP, corn RIP, rye
RIP and flax RIP; the bacterial toxins: diptheria
toxin, pseudomonas exotoxin and shiga toxin and
chemical toxins: melphalan, methotrexate, nitrogen
mustard, doxorubicin and daunomycin.
20   Again, the most preferred forms of these compounds
will have the toxin T group (or T groups) attached to
the linkage group Y at the site of an amino group, a
carboxylic group or a sulfhydryl group of the Y group.
In other words the T group will be attached at an
25   amino, carboxylic or sulphydral group of the
carbodiimide, the 2-iminothiolane, SPDP, bis-
diazobenzidine or the glutaraldehyde which is used as
the linkage group Y. Similarly, the Y group will be
attached to the X group at the site of an amino group,
30   a carboxylic group or a sulphydral group of the amino
acid group which is employed as the X group of the
overall compound.
           Some of the more preferred compounds made
according to this patent disclosure are based upon the
35   following additional, preference considerations

regarding compounds comprised of a hormone group, a
linking agent group and a toxin group. The most
pref rred hormones that may b used to produce our
hormonotoxins for chemical castration would include:

5   (1)   (pyro)Glu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-
Ethylamide   D-Lys^5-GnRH-Pro^9-Ethylamide,   (2)
luteinizing hormone (LH) or (3) interstital-cell
stimulating hormone (ICSH).   Similarly, the most
useful linking agents to produce hormonotoxins would

10   include: (1)   Succinimidyl   4-(p-maleimidophenyl)
butyrate (SPDP) and (2) N-Succinimidyl 3-(2-pyridyl-
dithio) proprionate (SMPB).   Finally, the most
promising toxins for production of hormonotoxins would
include (1)   Diphtheria toxin A chain and (2)

15   Pseudomonas exotoxin A chain.   Within the possible
compounds which could be made by combining the above
hormones, linking agents and toxins, two compounds are
especially preferred.   They are: (1) D-Lys^6-GnRH-
SMPB-diphtheria toxin A chain conjugate and (2) D-

20   Lys^6-GnRH-SMPB-speudomonas exotoxin A chain conjugate.
It should also be noted in passing that the toxins can
either be derived from natural sources or produced
using recombinant DNA technology.

Applicants have found that GnRH conjugations of

25   the types noted in TABLE II above are particularly
effective in causing the toxic compound T to be
specifically targeted to the gonadotropin-secreting
cells of the anterior pituitary gland. Indeed they
are the only cells to which the gonadotropin-releasing

30   hormone portion of the conjugate will bind. Hence,
these toxic compounds, bound to an analog of
gonadotropin-releasing hormone, can be employed to
permanently destroy a subpopulation of the anterior
pituitary cells and thereby eliminate the gland's

35   ability to secrete gonadotropins. This in turn causes

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the animal's gonads t atrophy and lose their ability
to function for reproductive purposes. That is to say
that, without functioning gonadotrophs, an animal is
not able to secrete luteinizing hormone (LH) and

5   follicle-stimulating hormone (FSH) and thus is
rendered sterile. Applicants have postulated that the
compounds of this patent disclosure inhibit synthesis
of LH, and presumably other proteins made by
gonadotrophs, because they tend to inhibit all protein

10   synthesis once these compounds gain entry into a cell.
It should also again be noted that applicants'
compounds act as GnRH superagonist. Hence, use of
applicants' compounds allows "chemical castration" to
be employed in place of surgical castration.

15   Thus, the use of these compounds has great utility
in human medicine as well as in veterinary medicine.
This follows from the fact that there are several
important biological reasons for employing castration
and antifertility drugs in humans. For example,

20   breast and prostate cancers are but two examples of
sex steroid-dependent tumors which respond to such
hormonal manipulation. At present, the only reliable
way to inhibit steroid-dependent tumor growth is
through administration of counter-regulatory hormones

25   (e.g., DES in prostate cancer), sex-steroid hormone
binding inhibitors (e.g., tamoxifen in breast cancer)
or surgical castration. Thus the potential medical
uses of such chemical castration compounds are vast
and varied. For example, prostate cancer remains an

30   important cause of cancer deaths and represents the
second leading cancer of males. The present
palliative treatment for advanced prostate cancer
cases involves reduction of serum testosterone/DHT
levels through use of surgical castration. It should

35   also be noted that for purposes of disease and/or

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fertility control, especially in humans, it may be
desirable to use applicants' compounds to ablate
pituitary gonadotr phs in conjunction with other modes
of treatment. For example, it is anticipated that

5   chronic administration of progestins and estrogens to
females and androgens to males might be necessary to
prevent loss of secondary sex characteristics,
behavior and osteoporosis. However, through judicious
use of the herein disclosed compounds, especially in

10   combination with appropriately administered sex
steroids, desirable antifertility effects can be
achieved. Another area of application in human
medicine is treatment of endometriosis. This
condition, which produces painful growth of

15   endometrial tissue in the female peritoneum and pelvis
also responds to inhibition of sex steroid synthesis.
Those skilled in this art will also appreciate that
the herein disclosed compounds could be used to
partially reduce sex-steroid secretions, and thus

20   reduce or eliminate certain hormone related behavior
problems while retaining improved growth stimulation.
     The dose/time adjustments associated with the use
of these compounds can vary considerably; however,
these compounds are preferably administered by

25   injection into a mammal in concentrations of from
about 0.1 to about 10 milligrams per kilogram of the
mammal's body weight. Sterilization may be
accomplished with as few as one injection; but
multiple treatments (e.g., based upon concentrations

30   of from about 0.03 milligrams once every 4 days to
about 1 milligram per kilogram of body weight for 20
days) are alternative sterilization schemes.
Furthermore, as sterilization agents, the compounds of
this patent disclosure can be used before or after

35   puberty. They too are especially useful in those

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areas f animal husbandry wher th anabolic benefits of non-surgical sterilization techniques can contribute to meat production and/or quality. In one preferred embodiment of this invention the compounds

5 of this invention are administered to male cattle between the ages of about 8 weeks and 20 weeks at least once and in a concentration of from about 0.1 to about 10 milligrams per kilogram of the animal's body weight.

10 The toxic moieties T of the herein disclosed compounds are obtainable from both natural and synthetic sources. For example, pokeweed antiviral protein can be isolated from leaves of pokeweed plants and purified by gel filtration chromatography. It can

15 then be, by way of example, conjugated to $D-Lys^6-$ $desGly^{10}-pro^9GnRH$-ethylamide via the amino group on the lysine and through a sulfhydryl group introduced into the pokeweed antiviral protein by a heterobifunctional reagent. In any event, one of the chief advantages of

20 these compounds is their ability to produce permanent sterilization without strong toxic side effects. Hence these compounds may be used on mammals such as human beings, domestic animals, pets or wild animals. Moreover, they can be administered as a single

25 injection which can induce permanent and irreversible sterility in both male and female mammals. However, an alternative approach to achieve sterilization is through multiple injections at lower dosages than those employed in a single treatment or by slow

30 release implants (i.e., biodegradable formulations). Applicants also have postulated that the "B-chain" portion of their toxic moieties are important not only for binding to cell surfaces, but for trans-membrane translocation of their A-chain. This was particularly

35 demonstrated for the A-chain of Diphtheria toxin,

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Ricin and Pseudomonas exotoxin. To this end,
applicants prepared conjugates of GnRH-A to A and B
chains of Diphtheria t xin as well as to a modified A-
B chain which was genetically engineered to eliminate
the carboxy terminal binding portion of the B-chain.
These conjugates were shown to bind to pituitary cell
GnRH receptors. They were found to possess enhanced
toxicity over A-chain conjugates based on improved
trans-membrane transport characteristics. Given this,
those skilled in the art will appreciate that numerous
genetic and chemical modifications of B-chains should
allow further exploitation of this approach. That is
to say that, by such methods, it is possible to
generate a whole series of conjugates that can be
characterized as GnRH-A-A/B, GnRH-A-A, GnRH-A-A plus
GnRH-B, all of which could enhance the findings
described herein by simultaneous delivery of membrane
active B-chains with the herein described GnRH-A-T
conjugates.

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DESCRIPTION OF DRAWINGS

Figures 1A and 1B respectively depict the results of GnRH induced secretion of LH based upon a singl injection of a GnRH-A-T compound and the results of GnRH induced secretion of LH based upon 4 injections of a GnRH-A-T compound.

Figure 2 indicates inactivation of certain grain hemitoxins (wheat hemitoxin and barley hemitoxin) by SPDP conjugation.

Figure 3 depicts the results of a SDS-PAGE analysis of carbodiimide conjugated hemitoxins.

Figure 4 shows the inhibition of 2-iminothiolane-conjugated barley hemotoxin.

Figure 4A shows SDS-PAGE analysis of barley hemitoxin after conjugation to [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide using 2-iminothiolane.

Figure 5 shows binding curves indicating the ability of [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide toxin conjugates to bind to pituitary receptors.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

One of the chief objects of this invention is to provide a class of compounds which will allow safe, inexpensive, chemical castration. As such, applicants' compounds represent an alternative to surgical castration as well as to surgery for treatment of diseases such as breast cancer or certain sex hormone related prostate cancer. In order to better define this class of compounds, Applicants conducted studies on various linking technologies as they apply to numerous toxin candidates. These studies resulted in the herein disclosed group of conjugate compounds. In general these compounds display exceptional gonadotroph membrane binding characteristics along with full retention of toxin

activity.

In general, the sterilization activity of the
compounds of this patent disclosure was tested in
receptor binding assays (to be sure a given conjugate
5   was still capable of interacting with the GnRH
receptor cells of the pituitary), in a cell-free
translation system (to insure that the toxic protein
maintained its toxicity), in cell culture systems (to
determine if a given toxic conjugate is capable of
10   inhibiting synthesis of LH), and in test animals (to
determine if sterility was induced). The most
effective of these sterilization agents found to this
point in Applicants' research is D-Lys^6-desGly^{10}-pro^9-
GnRH-ethylamide conjugated to pokeweed antiviral
15   protein using carbodiimide as the linkage between the
carrier protein molecule and the toxin moiety.

Again, a distinct advantage of each of the
sterilization agents of this invention, and pokeweed
antiviral protein in particular, is that they have an
20   extremely limited ability to enter cells in an
animal's body unless they are first conjugated to a
carrier such as gonadotropin-releasing hormone. Such
conjugation can be accomplished in several ways. By
way of example, pokeweed antiviral protein can be
25   conjugated to a [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide
molecule via the ϵ-amino group on the Lysine and
through a sulfhydryl group on the pokeweed antiviral
protein.

By way of example, applicants found that this type
30   of linkage reduces the ability of the [D-Lys^6, des-
Gly^{10}]-GnRH-ethylamide to bind to the GnRH receptor by
99%. In addition, the conjugation procedure reduces
the toxicity of the pokeweed antiviral protein by 99.5
in a cell-free translation system. However, despite
35   large reductions in activity of both the GnRH analog

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and the st rilization agent by this particular
conjugation procedure, some activity of each was
maintained. The activity of this conjugate was also
tested in a pituitary cell culture system. In this
5   system, pituitary cells were incubated with the
sterilization agent conjugated to [D-Lys^6, des-Gly^{10}]-
GnRH-ethylamide for 16 hours. After incubation, the
sterilization agents were removed from the incubation
media by extensive washing and the cells were then
10   cultured for an additional 24 hours. The increase in
total LH, i.e., that present in the media plus that in
the cells during the 24 hour period, represents the
ability of the treated cells to synthesize LH. Using
this system, it was established that these toxic
15   conjugates can completely inhibit synthesis of LH by
the cultured cells. Compared to control cells, the
sterilization agent alone reduced synthesis of LH by
only 20%. Thus, by this method, it was established
that the compounds of this patent disclosure can
20   inhibit synthesis of LH and presumably other proteins
made by gonadotrophs since this class of compounds has
the ability to inhibit all protein synthesis once they
gain entry into a cell.

Applicants also tested these compounds using an in
25   vivo model. The test system initially chosen was the
ovariectomized female rat. The parameter examined was
GnRH induced secretion of LH. The results of such an
experiment with rats are shown in Fig. 1A.   It
indicates that a single injection of a toxic conjugate
30   (i.e., GnRH-A-T) wherein the toxic moiety (T) pokeweed
antiviral protein and the GnRH-A moiety was [D-Lys^6,
des-Gly^{10}]-GnRH-ethylamide.   During week 1, this
compound induced secretion of LH equivalent to that of
GnRH-A alone. This indicated that the sterilization
35   agent conjugate was binding to the GnRH receptor in

vivo. During week 2, release of LH was reduced by 50%
in the GnRH-A treat d group (controls), but by >90% in
the GnRH-A-T group. By the third week, the release of
LH in the GnRH-A-T group had returned to the same

5   level as that observed in the control animals. This
indicated that a single treatment with the
sterilization agent conjugate was probably not
sufficient to completely kill the gonadotrophs in
vivo. It might however be the basis for a temporary

10   sterilization. Based upon this finding, a second
experiment was conducted to examine the effect of 4
injections of a pokeweed antiviral sterilization
conjugate at 3-day intervals on the ability of
ovariectomized rats to release LH. In this

15   experiment, the rats were unable to release LH in
response to GnRH stimulation one month after
initiation of the treatment (Fig. 1B). These data
strongly indicate the ability of these conjugates to
permanently inhibit reproduction in intact male and

20   female animals.

In another set of experiments, intact rats were
given 4 injections of GnRH-A-T compounds, again
wherein the toxic moiety T was selected from pokeweed
antiviral protein, ricin A chain, and ribosome

25   inhibiting proteins, of certain grains (again, those
of wheat, corn, barley and rye,) at 3-day intervals
and their subsequent reproductive capacity was
compared to rats treated with only the respective
toxin T or to that of untreated rats. In this

30   experiment, treatment of male rats with only the toxin
T did not reduce their fertility compared to controls
(percentage of females that became pregnant was 100%).
However, fertility was greatly reduced in those males
that were treated with a GnRH-A-T agent such as, for

35   example   D-Lys^6-desGly^{10}-pro^9-GnRH-ethylamide   con-

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jugated to pokeweed antiviral protein, i.e., only 50% of the females exposed to males became pregnant. Moreover, fertility did not appear to increase with time after treatment. Histological examination of the testes of these rats indicated that most of the seminiferous tubules were devoid of sperm. However, 10% of the tubules appeared to still be producing sperm and probably accounted for the pregnancies observed. The weight of the testes was reduced by nearly 50% and did not recover within 6 months after the end of treatment. Thus, the effects of the treatment appeared to be permanent and dose related. Female rats treated with the toxic conjugate were sterile and remained so for at least 4 months after the end of treatment. Most important is the fact that none of the rats treated with the toxic conjugate appeared to have any side effects.

EXEMPLARY CHEMICAL EXPERIMENTAL METHODS

1. Synthesis of [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide. Synthesis of this analogue was accomplished using the solid phase method on hydroxymethyl resin and cleavage from the resin by ethyl amine, yielding the ethylamide. Following HF cleavage of protecting groups from side chains the peptide was purified by countercurrent distribution, purity of the peptide was assured by TLC, paper electrophoresis, and amino acid analysis of the acid hydrolysate.

2. Applicants also produced a caproic acid derivative (134.91 mg) and the lysosomal hydrolase sensitive tetrapeptide spacer Leu-Ala-Leu-Ala-D Lys^6 (16.25 mg).

3. Conjugation of [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide to toxins using SPDP. Applicants

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endeavored to construct toxic conjugates of [D-Lys⁶, des-Gly¹⁰]-GnRH-ethylamide with the ricin A-chain. At the time these studies were initiated, Ricin A-chain was commercially available, but applicants found it to
5   be both expensive and very unstable to temperature changes or conjugation procedures. Construction of an effective hemitoxin [D-Lys⁶, des-Gly¹⁰]-GnRH-ethylamide conjugate requires coupling of hemitoxin to hormone via a protein cross-linking reagent that does not
10   block either the enzymatic activity cf the hemitoxin or the binding specificity of the hormone. Therefore, applicants investigated a number of different hemitoxins in addition to ricin A and pokeweed antiviral protein and a number of different
15   conjugation techniques. This work was largely directed at purification of certain plant hemitoxins, i.e., ribosomal inhibitory proteins, ("RIP"), a relatively recently recognized group of proteins which share the ability to enzymically inactivate mammalian
20   ribosomes. Such toxins are potentially promising as alternatives to the more familiar A-chains of, for example, ricin in that they do not require separation from the cell-binding B-chains. The bi-functional coupling reagent most commonly used for this purpose
25   is N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP). This compound forms covalent linkages to either free amino or sulfhydryl groups on proteins, but SPDP normally is attached to amino groups in hemitoxins, partly because many hemitoxins do not
30   contain sylfhydryls that are available for coupling.
       Initial experiments examined the reaction of SPDP with both the wheat and barley hemitoxins at various SPDP: hemitoxin ratios. The reactions were carried out at pH 9 for 30 minutes at 23°C at a protein
35   concentration of 0.6 mg/ml. After 30 minutes a 20-

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fold molar excess (over SPDP) of lysine was added to react with free SPDP and the hemitoxins diluted and assayed for inhibition of polyphenylalanine synthesis on Ehrlich ascites cell ribosomes. The results are
5   presented in Figure 2.

Figure 2 is intended to show inactivation of certain grain hemitoxins by SPDP conjugation. It indicates that even 1:1 ratios of SPDP to hemotoxin result in significant inactivation which is complete
10 at a 20:1 ratio. A commonly used 2-3 fold ratio would result in >95% inactivation. Applicants' study was expanded to include hemitoxins from corn and pokeweed. Reactions were carried out in phosphate buffers at neutral and acidic pH's in anticipation that under
15 acidic conditions differences in pKa of lysine amino groups. or conformational changes in some of the proteins might protect enzymic activity. However, in all conditions and with all 4 hemitoxin proteins, significant inactivation occurred and as quantitative
20 activity measurements of hemitoxins were rather imprecise; hence applicants were unable to conclude that residual activity was not from unreacted hemitoxin. Moreover, these particular experiments indicated SPDP would be unsuitable as a coupling
25 reagent for preparing many GnRH-A-T conjugates.

4.   Conjugation of [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide to toxins using Carbodiimide. Applicants examined the ability of the water soluble coupling reagent, carbodiimide linkages in this class of
30 compounds. Although carbodiimide has been used successfully for coupling polypeptide hormones to proteins, applicants are unaware of any studies reporting its use in preparing toxin-protein conjugates. However, its use turned out to be
35 attractive since it couples through carboxyl groups on

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the hemitoxin rather than amino groups. It should also be noted that applicants' synthetic GnRH analogs are blocked at the carboxyl and amino termini, thus leaving, for example, D-lys^6 amine as the only reactive moiety. Use of large molar ratios of GnRH favors reaction of the hemitoxin to the analog rather than to itself.

Figure 3 shows the successful results of this approach. It represents a SDS-PAGE analysis of carbodiimide conjugated hemitoxins. In order to carry out these experiments, a 30:1 molar ratio of D-Lys^6, des-Gly^{10}] – GnRH – ethylamide to hemitoxin was reacted with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) in water at 23°C for 30 minutes and the reaction mixture passed through a Bio-Gel P6 column to desalt the product. Protein containing fractions were assayed for residual activity (see text) and the reaction products examined by SDS polyacrylamide gel electrophoresis. Lanes 1, and 6 are standards; lane 2, barley; lane 3, barley-GnRH; Lane 4, pokeweed; lane 5, pokeweed-GnRH; lane 7, rye-GnRH; lane 8, rye; lane 9, gelonin-GnRH; lane 10, gelonin. Conjugation in each case resulted in a 32kDa product which was distinct from the 30 kDa hemitoxin alone, and which (by enzyme assay) retained 10% of the original activity. Hemitoxins from barley, rye, wheat and the unrelated pokeweed and gelonin hemitoxins have each been successfully conjugated in this fashion and all retain about 10% of original toxicity in ascites ribosomal assay. Biologic studies with these conjugates were then completed in the manner hereinafter described.

5. Conjugation of [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide to toxins using 2-iminothiolane. Although 2-iminothiolane, like SPDP, reacts with free amino

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groups on proteins, it does not affect the activity of
gelonin or PAP. Applicants have hypothesized that
perhaps the reason 2-iminothiolane differs from SPDP
in this regard is that it reacts with a different
5   amino group on the protein or that it places a
positive charge on the active amino group and thereby
preserves enzymatic activity. In any case, applicants
reacted 2-iminothiolane with barley hemotoxin at
several reagent: protein ratios, separated the protein
10   from unreacted 2-iminothiolane by gel exclusion
chromatography on Sephadex G-25 and quantitated the
amount of sulfhydryl groups introduced onto the
hemitoxin by sulfhydryl exchange with the reactive,
chromogenic disulfide 5,5'-dithiobis (2-nitrobenzoic
15   acid) (DTNB). The derivatized barley hemitoxin
preparations were assayed for their ability to inhibit
protein synthesis in ascites cell-free extracts and
were found to have retained full activity.

Figure 4 depicts inhibition of protein synthesis
20   by 2-iminothiolane-conjugated barley hemitoxin.
Barley hemitoxin was incubated at 0°C for 90 minutes
with 0 (o), 8-fold (x) or 24-fold (o) molar excess of
2-iminothiolane. The derivatized hemitoxins were then
assayed for their ability to inhibit protein synthesis
25   in ascites cell-free extracts. Proteins contained 0
(o), 0.76 (x) and 1.44 (o) moles of 2-iminothiolane
bound per mole of hemitoxin.

Conjugation between the barley hemitoxin and [D-
Lys^6, des-Gly^{10}]-GnRH-ethylamide was carried out by
30   disulfide exchange. A sulfhydryl group was introduced
into [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide by reacting
the hormone with a 16- fold molar excess of 2-
iminothiolane at 0°C for 2 hours. Derivatized [D-
Lys^6, des-Gly^{10}]-GnRH-ethylamide was separated from
35   unreacted 2-iminothiolane by chromatography on a Bio-

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Gel P-2 column equilibrated with 30% acetic acid. Acetic acid was removed from the isolated hormone by rotary evaporation followed by lyophilization. A reactive disulfide was prepared from barley hemitoxin
5   as described above by incubating the hemitoxin with a 24-fold molar excess of 2-iminothiolane, isolating the protein and reacting it with DTNB to prepare the disulfide, and separating the hemitoxin from unreacted DTNB by column chromatography on Sephadex G-25. A 12-
10   fold molar excess of derivatized [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide was added to hemitoxin disulfide and disulfide exchange permitted to occur overnight at 4°C. Hemitoxin was separated from unconjugated GnRH by Sephadex G-25 column chromatography.

15   The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis under non-reducing conditions. Analysis showed that the coupling reaction had converted approximately 50% of the 29 kDa barley hemitoxin (track 5) into a 31 kDa product
20   (tracks 1-4) corresponding to a 1:1 hemitoxin- [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide conjugate. The faint band of unreacted [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide that can be seen in track 1 migrating ahead of the 14 kDa marker disappeared following acetone precipitation
25   of the hemitoxin (track 2) or gel exclusion chromatography on Sephadex G-25 (tracks 3 & 4). The mixture of conjugate and unreacted hemitoxin was not purified further but was assayed directly for pituitary cell binding and killing.

30   Figure 4A depicts SDS-PAGE analysis of barley hemitoxin after conjugation to [D-Lys^6, des-Gly^{10}]-GnRH -ethylamide using 2-iminothiolane. Reaction products were analyzed before (tracks 1 & 2) and after tracks 3 & 4) Sephadex G-25 chromatography, and before
35   (tracks 1 & 3) and after (tracks 2 & 4) concentrating

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by acetone precipitation. Track 5 contained unreact d hemitoxin.

6. Conjugate Binding Studies. In order to assess whether [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide toxin conjugates retain their ability to bind to receptors, the following assay was devised. Various concentrations of each conjugate were evaluated for their ability to displace 50,000 cpm ^{125}I-D Ala^6-GnRH from bovine pituitary membranes. After incubation for 4 hours in standard conditions at 4°C, membranes were pelleted, counted in a gamma counter to determine the bound labelled ligand, and the ability of each conjugate to displace 50% of the label (IC$_{50}$ for unlabelled [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide. Figure 5 indicates the results of binding curves obtained in these experiments. Also shown are the calculated number of molecules required to displace 1 molecule of unconjugated [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide. For example, Figure 5 shows competitive binding of toxin conjugates to bovine pituitary membranes. The abbreviations are: 2IT, 2-iminothiolane; PAP, Pokeweed Antiviral Protein; SPDP, N-succinimidyl 3-(2-pyridyldithio) propionate; CI, Carbodiimide; EACA, Epsilon-amino caproic acid linker. Grain names refer to the purified hemotoxin source.

The data in Figure 5 was critical in determining applicants' next steps. Several conclusions were reached. First, SPDP severely limits toxin activity (see Figure 2). It also produces conjugates with greatly reduced binding activity (compare PAP-SPDP with Barley carbodiimide). On the other hand, use of carbodiimide produced conjugates with 3-40 fold improved binding compared to SPDP. However, there were differences among the hemitoxins used. For example, the wheat, rye and gelonin carbodiimide

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conjugates all showed greater binding than did the barley carbodiimide conjugate. However, the barley carbodiimide conjugate retained greater toxicity than the other grain hemitoxin conjugates in the cell free protein synthesis assay (data not shown). In this case, use of a spacer arm actually decreased binding affinity. Finally, the 2-iminothiolane conjugate made with barley hemitoxin as described above retained both 100% toxicity in the cell free system (see generally Figure 4) and was as active as the best of the carbodiimide conjugates in binding. Applicants noted a 4.5 fold reduction in binding compared to the unconjugated [D-Lys^6, desGly^{10}]-GnRH-ethylamide. This was quite acceptable since native GnRH has also only about 1/30 the binding activity as this analogue (data not shown). Thus, after this exploratory work was completed, applicants carried out most further work with either the PAP-SPDP-[D-Lys^6, desGly^{10}]-GnRH-ethylamide or the barley 2-imminothiolane [D-Lys^6, desGly^{10}]-GnRH-ethylamide conjugate.

In Vitro Experiments.

The effect of these compounds on ovine pituitary cells in suspension culture was measured. A pituitary was removed from a ewe, sliced thinly, and dissociated with a mix of collagenase, hyaluronidase, and DNAase. The cells were washed several times and resuspended in culture medium containing 30% ram's serum. Cells were cultured in a 37° shaking water bath in 50 ml flasks under 95% O_2/5% CO_2. In a typical experiment, cells were divided into four groups after dissociation and cultured overnight (20 hr) with 1) culture medium only, 2) 10^{-8} M GnRH, 3) 3×10^{-9}M Toxin-[D-Lys^6, desGly^{10}]-GnRH-ethylamide (molarity expressed in terms of GnRH receptor binding activity) and 4) Toxin at the same concentration as Toxin-[D-Lys^6, desGly^{10}]-GnRH-

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ethylamide. After pretreatm nt, the cells were washed
6 times, counted, and small aliquots removed for
testing. The remainder were cultured in plain m dium
for 24 hours. To test the cells, aliquots of 500,000
cells were washed and resuspended in challenge medium
containing 10^{-7}M GnRH for 2 hours at 37°C. 3 ml of
cold Gel-PBS was added to each tube, cells were
centrifuged, and the media was measured for LH
content. The four pretreatment groups were evaluated
for their ability to synthesize and secrete LH
immediately after treatment and after the 24 hour
recovery period. The results of one experiment are
shown in Table III.

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TABLE III

LH Synthesis and Release by Ovine Pituitary Cells
(ng per 5 x 10^6 cells)

TREATMENT[1]	SYNTHESIS[2]	I N D U C E D RELEASE[3]
CONTROL	526.3	723.9
10^{-8} M GnRH	545.5	320.3
PAP	137	26.2
PAP-D-Lys[6]	0	11.7

[1] Cells were incubated with the various treatments for 16 hours.

[2] Synthesis of LH was measured during a 24 hour period of culture after the agents were removed from the cells.

[3] Release of LH was induced by maximal dose of GnRH (10^{-7} M).

These data, although obtained with the least promising of our conjugates, reveal a large and specific effect of PAP-SPDP-[D-Lys[6], desGly[10]]-GnRH-ethylamide (ethylamide is abbreviated as "EA" in Table III) on the gonadotropes ability to synthesize and secrete LH. It is not possible to determine whether the gonadotropes were specifically killed as they comprise <10% of the total number of pituitary cells, but the data strongly suggest the conjugate disrupted their normal function.

Applicants then tested the more promising Barley-2IT-[D-Lys[6], desGly[10]]-GnRH-ethylamide conjugate in similar assay systems. Table IV shows the results of a similar experiment. Ovine pituitary cells were again placed in culture with various agents and the

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total LH in the cells and media determin d after a 24
hour exposure, wash, and further 24 hour culture in
standard media.

TABLE IV

Total Culture LH after Exposure to GnRH
and Toxin Conjugates with or without Lysosomal Agents

Incubation Condition	Total LH($Ng/10^5$ cells in Culture)
Control	1.90
D-Lys^{6}GnRH-EA	1.62
Barley Toxin	1.49
Barley Toxin-2IT-D-Lys^{6}GnRH-EA	.91
Barley Toxin-2IT-D-Lys^{6}GnRH-EA + Monensin	1.83
Barley Toxin-2IT-D-Lys^{6}GnRH-EA + Chloroquine	.62
Barley Toxin-2IT-D-Lys^{6}GnRH-EA + NH$_4$Cl	1.33
Barley Toxin-2IT-D-Lys^{6}GnRH-EA + Killed Adenovirus	1.13

These results indicate a specific killing effect of
the toxin conjugate after only 24 hours of exposure.
The lysosomally active agents do not potentiate this
effect with the exception of chloroquine. When such
experiments are combined with secondary challenge by
GnRH, it appears that few gonadotropes are able to
synthesize new GnRH after exposure to the barley toxin
conjugate (data not shown).

7. In vivo Experiments. Several experiments were
done to determine the effects of the pokeweed toxin
(PAP)-SPDP-[D-Lys^6, desGly^{10}]-GnRH-ethylamide conjugate
in adult Sprague Dawley rats. Groups of 5-7 rats were
treated with 20 ng of analogue; 20 ng conjugate
(receptor binding assay equivalents), saline, or a
conjugate made from a protein of similar molecular
weight to the pokeweed toxin (carbonic anhydrase or
ovalbumin). The most effective time course was found
to be weekly injections for 4 weeks. The effect of

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such treatment was monitored in several ways. The
ability of th animals to respond to a GnRH analogue
challenge by measuring LH and/or serum testosterone
levels 30-90 minutes after injection was followed. No
5   difference was found among the groups. This result
might be expected, since inducible LH release in
intact animals is quite small secondary to chronic
feedback suppression by the testicular androgens.
Secondly, applicants followed gonad weights and found
10   the   testes   in   the   PAP-[D-Lys^6, desGly^{10}]-GnRH-
ethylamide group to be decreased by 50%, although the
control conjugates had similar effects. The PAP and
carbonic anhydrase conjugate groups were found to be
infertile in breeding tests, indicating a potential
15   effect   of   this   enzyme   on   testis   tissue.
Interestingly, light microscopy of these animals
revealed no changes in the pituitaries, but
interstitial (Leydig) cell depletion in the PAP-SPDP-
[D-Lys^6, desGly^{10}]-GnRH-ethylamide treated group,
20   indicating a possible specific cellular effect on rat
testicular function. This was not surprising since
there are GnRH receptors on Leydig cells in the rat
testis.

Applicants also tested the PAP-carbodiimide-[D-
25   Lys^6, desGly^{10}]-GnRH-ethylamide   conjugate   in
ovariectomized female rats. In contrast to the SPDP
conjugate, and in this system where gonadal feedback
is not a problem, this drug appears capable of
producing a 15 fold decrease in the serum LH response
30   to GnRH analogue challenge (Figure 1A or 1B), again
indicating the importance of applicant's studies on
various linking techniques.

Figure 1B indicates the results of a challenge by
one of applicants' compounds to ovariectomized rats.
35   Serum concentrations of LH in ovariectomized rats

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treated with saline (hatched bars) or pokeweed anti-
viral protein conjugated to a GnRH super-agonist
(solid bars) are d picted. Th open space above the
bars indicates the amount of LH released in response
5   to a GnRH challenge. The challenges were administered
on the first day of treatment and again 4 weeks later.
Compared to control there was greater than a 90%
reduction in LH release after GnRH challenge at 4
weeks of treatment.

10   Based on the above data (with regards to LH
synthesis inhibition) applicants then carried out
experiments in intact male and female rats. Animals
received 4 injections at 3 day intervals of PAP-CI-[D-
Lys^6, desGly^{10}]-GnRH-ethylamide or of the GnRH analogue
15   or toxin alone or saline. Conjugate treated male
animals (but not control) showed a 50% reduction in
fertility (i.e., 50% of females exposed to these male
animals became pregnant, compared to 100% of
controls). Histologic examination of the testes of
20   experimental animals revealed residual spermatogenesis
in about 10% of tubules. In conjugate treated female
animals, fertility was abrogated for more than 4
months following treatment. There were no side
effects noted from these injections.

25   To further understand the effect of hemitoxins and
conjugates on non-target tissues, applicants initiated
studies on the tissue distribution of ^{125}I-toxin-
conjugates and have demonstrated important differences
among the toxins in (for example) concentration in the
30   kidneys, indicating the importance of testing the
various proteins to avoid potential non-target tissue
toxicity. For example, applicants have found that the
tissue/serum ratio of unconjugated PAP 2 hours after
injection for various organs ranges from .03 in brain
35   to 85.5 in kidney. In contrast, unconjugated barley

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hemotoxin is 8-fold less concentrated in kidney (see Table IV). Conjugation with the GnRH analogue alters these ratios consid rably.

TABLE V

Tissue Distribution of Hemitoxins
and Hemotoxin Conjugates

Tissue	PAP	PAP-SPDP-D-Lys^{6}GnRH
Pituitary	.20	.11
Brain	.03	.01
Adrenal	.48	.02
Kidney	85.5	12.6
Liver	2.48	1.07
Spleen	2.29	.73
Testis	.03	.02

Tissue/Serum Ratio of Labeled Protein

GnRH	Barley	Barley-CI-D-Lys^{6}GnRH
Pituitary	1.08	1.06
Brain	.04	.04
Adrenal	.70	1:5
Kidney	10.5	4.0
Liver	.43	3.52
Spleen	.4	5.07
Testis	.10	.10

Thus these experiments produced a group of compounds capable of sterilizing (temporarily or permanently) animals by destroying the gonadotrophs of an animal's anterior pituitary gland. These compounds can be administered in the form of pharmaceutically acceptable, and otherwise nontoxic salts. It should also be noted that these compounds can be administered individually, or in combination with each other, to animals intravenously, subcutaneously, intramuscularly or orally to achieve fertility inhibition and/or control. Preferably administration will be intravenous or intramuscular in a suitable carrier

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such as, for exampl , in isotonic saline phosphate
buffer s lutions or the like. They also can be used
in applications calling for reversible suppression of
gonadal activity, such as for the management of
5 precocious   puberty   or   during   radiation   or
chemotherapy. Effective dosages will vary with the
form of administration and the particular species of
mammal being treated. An example of one typical
dosage form is a physiological saline solution
10 containing the peptide which solution is administered
to provide a dose in the range of about 0.1 to 10
mg/kg of body weight.

    Although the invention has been described with
regard to its preferred embodiments, it will be
15 apparent to those skilled in this art, upon reading
the above detailed description and examples, that
various modifications and extensions can be made
thereto without departing from the spirit of the
present invention and that the scope of said invention
20 shall be limited only by the scope of the appended
claims.

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Thus having disclosed our invention, we claim:

1. A method for sterilizing an animal, said method comprising administering an effective amount of a compound comprised of a peptide hormone group

5   capable of binding with a receptor cell of the animal's pituitary gland and wherein said peptide hormone is selected from the group consisting of a GnRH analog (GnRH-A), human chorionic gonadotropin, equine chorionic gonadotropin, luteinizing hormone and

10   follicle - stimulating hormone, which peptide hormone is conjugated to a toxin group capable of destroying said receptor cell of the pituitary gland when said toxin group is conjugated to said peptide hormone.

2. The method of claim 1 wherein the animal

15   is selected from the group of animals consisting of mammals, fowl and fish.

3. A method for sterilizing an animal, said method comprising administering an effective amount of a compound comprised of a peptide hormone group

20   capable of binding with a receptor cell of the animal's pituitary gland and wherein said peptide hormone is selected from the group consisting of a GnRH analog (GnRH-A), human chorionic gonadotropin, equine chorionic gonadotropin, luteinizing hormone and

25   follicle - stimulating hormone, which peptide hormone is conjugated to a toxin group capable of destroying said receptor cell of the pituitary gland and wherein said toxin group is selected from the group consisting of the plant toxins: ricin, modeccin, abrin, pokeweed

30   anti-viral protein, α-amanitin, gelonin ribosome inhibiting protein ("RIP") barley RIP, wheat RIP, corn RIP, rye RIP and flax RIP; the bacterial toxins: diptheria toxin, pseudomonas exotoxin and shiga toxin

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and the chemical toxins: m lphalan, methotrexate,
nitrogen mustard, doxorubicin and daunomycin.

        4.   The method of claim 3 wherein the animal
is selected from the group of animals consisting of
mammals, fowl and fish.

        5.   A method for sterilizing an animal, said
method comprising administering an effective amount of
a compound having the formula:

        pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Z
                                      |
                                      X
                                      |
                                      Y
                                      |
                                      T

wherein X is an amino acid selected from the group
consisting of tyrosine, D-tyrosine, ornithine, D-
ornithine, glutamic acid, D-glutamic acid, aspartic
acid, D-aspartic acid, cysteine and D-cysteine; Y is
a linking agent selected from the group consisting of:
carbodiimide, 2-iminothiolane, SPDP, (N-succinimidyl
3-(2-pyridyldithio) propionate), bis-diazobenzidine
and glutaraldehyde; Z is a chemical group selected
from the group consisting of Gly-NH$_2$, ethylamide, and
Aza-Gly-NH$_2$ and T is a toxin group selected from the
group consisting of the plant toxins: ricin, modeccin,
abrin,   pokeweed   anti-viral   protein,   α-amanitin,
gelonin ribosome inhibiting protein ("RIP") barley
RIP, wheat RIP, corn RIP, rye RIP and flax RIP; the
bacterial   toxins: diptheria   toxin,   pseudomonas
exotoxin and shiga toxin and the chemical toxins:
melphalan, methotrexate, nitrogen mustard, doxorubicin
and daunomycin.

6.   A method for treating sex horm ne r lated
diseas s of a mammal, said method comprising
administering an effective amount of a compound
comprised of a peptide hormone group capable of
5   binding with a receptor cell of the mammal's pituitary
gland and wherein said peptide hormone is selected
from the group consisting of a GnRH analog (GnRH-A),
human chorionic gonadotropin, equine chorionic
gonadotropin, luteinizing hormone and follicle -
10   stimulating hormone, which peptide hormone is
conjugated to a toxin group capable of destroying said
receptor cell of the pituitary gland when said toxin
group is conjugated to said peptide hormone.

7.   A method for treating sex hormone related
15   diseases of a mammal, said method comprising
administering an effective amount of a compound
comprised of a peptide hormone group capable of
binding with a receptor cell of the mammal's pituitary
gland and wherein said peptide hormone is selected
20   from the group consisting of a GnRH analog (GnRH-A),
human chorionic gonadotropin, equine chorionic
gonadotropin, luteinizing hormone and follicle -
stimulating hormone, which peptide hormone is
conjugated to a toxin group capable of destroying said
25   receptor cell of the pituitary gland and wherein said
toxin group is selected from the group consisting of
the plant toxins: ricin, modeccin, abrin, pokeweed
anti-viral protein, α-amanitin, gelonin ribosome
inhibiting protein ("RIP") barley RIP, wheat RIP, corn
30   RIP, rye RIP and flax RIP; the bacterial toxins:
diptheria toxin, pseudomonas exotoxin and shiga toxin
and the chemical toxins: melphalan, methotrexate,
nitrogen mustard, doxorubicin and daunomycin.

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8.   A method for treating sex hormone related diseases of a mammal, said method comprising administering an effective amount of a compound having the formula:

5   pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Z
                                                |
                                                X
                                                |
                                                Y
10 |
                                                T

wherein X is an amino acid selected from the group consisting of tyrosine, D-tyrosine, ornithine, D-ornithine, glutamic acid, D-glutamic acid, aspartic
15   acid, D-aspartic acid, cysteine and D-cysteine; Y is a linking agent selected from the group consisting of: carbodiimide, 2-iminothiolane, SPDP, (N-succinimidyl 3-(2-pyridyldithio) propionate), bis-diazobenzidine and glutaraldehyde; Z is a chemical group selected
20   from the group consisting of Gly-NH$_2$, ethylamide, and Aza-Gly-NH$_2$ and T is a toxin group selected from the group consisting of the plant toxins: ricin, modeccin, abrin, pokeweed anti-viral protein, α-amanitin, gelonin ribosome inhibiting protein ("RIP") barley
25   RIP, wheat RIP, corn RIP, rye RIP and flax RIP; the bacterial toxins: diptheria toxin, pseudomonas exotoxin and shiga toxin and the chemical toxins: melphalan, methotrexate, nitrogen mustard, doxorubicin and daunomycin.

30   9.   A method for increasing the rate of protein formation on an animal raised for its meat, said method comprising administering an effective amount of a compound having the formula:

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$$pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Z$$
$$|$$
$$X$$
$$|$$
$$Y$$
$$|$$
$$T$$

wherein X is an amino acid selected from the group
consisting of tyrosine, D-tyrosine, ornithine, D-
ornithine, glutamic acid, D-glutamic acid, aspartic
acid, D-aspartic acid, cysteine and D-cysteine; Y is
a linking agent selected from the group consisting of:
carbodiimide, 2-iminothiolane, SPDP, (N-succinimidyl
3-(2-pyridyldithio) propionate), bis-diazobenzidine
and glutaraldehyde; Z is a chemical group selected
from the group consisting of Gly-NH$_2$, ethylamide, and
Aza-Gly-NH$_2$ and T is a toxin group selected from the
group consisting of the plant toxins: ricin, modeccin,,
abrin, pokeweed anti-viral protein, α-amanitin,
gelonin ribosome inhibiting protein ("RIP") barley
RIP, wheat RIP, corn RIP, rye RIP and flax RIP; the
bacterial toxins: diptheria toxin, pseudomonas
exotoxin and shiga toxin and the chemical toxins:
melphalan, methotrexate, nitrogen mustard, doxorubicin
and daunomycin.

10.   The method of claim 9 wherein the animal
is selected from the group of animals consisting of
mammals, fowl and fish.

11.   A compound, used for destroying
gonadotrophs of the pituitary gland, said compound
having the formula:

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pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Z
|
X
|
Y
|
T

wherein X is an amino acid selected from the group consisting of tyrosine, D-tyrosine, ornithine, D-ornithine, glutamic acid, D-glutamic acid, aspartic acid, D-aspartic acid, cysteine and D-cysteine; Y is a linking agent selected from the group consisting of: carbodiimide, 2-iminothiolane, SPDP (N-succinimidyl 3-(2-pyridyldithio) propionate), bis-diazobenzidine and glutaraldehyde; Z is a chemical group selected from the group consisting of Gly-NH_2, ethylamide, and Aza-Gly-NH_2 and T is a toxin group selected from the group consisting of the plant toxins: ricin, modeccin, abrin, pokeweed anti-viral protein, α-amanitin, gelonin ribosome inhibiting protein ("RIP") barley RIP, wheat RIP, corn RIP, rye RIP and flax RIP; the bacterial toxins: diptheria toxin, pseudomonas exotoxin and shiga toxin and the chemical toxins: melphalan, methotrexate, nitrogen mustard, doxorubicin and daunomycin.

12. The compound of claim 11 where the toxin group T is attached to the linkage group Y at a point on the Y group which comprises a chemical group selected from the group consisting of an amino group, a carboxylic group or a sulfhydryl group.

13. The compound of claim 11 wherein the linkage group Y is attached to the amino acid group X at a point on the amino acid group X which comprises a chemical group selected from the group consisting of

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an amino group, a carboxylic group or a sulfhydryl group.

14. A compound used for destroying gonadotrophs of the pituitary gland, wherein said compound is comprised of a hormone selected from the group consisting of (pyro)Glu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Ethylamide D-Lys^5-GnRH-Pro^9-Ethylamide, a luteinizing hormone or an interstital-cell stimulating hormone, a linking agent selected from the group consisting of succinimidyl 4-(p-maleimidophenyl) butyrate (SPDP) and N-Succinimidyl 3-(2-pyridyldithio) proprionate (SMPB) and a toxin selected from the group consisting of diphtheria toxin A chain and pseudomonas exotoxin A chain.

15. A compound used for destroying gonado-trophs of the pituitary gland, said compound being a D-Lys^6-GnRH-SMPB-diphtheria toxin A chain conjugate.

16. A compound used for destroying gonado-trophs of the pituitary gland, said compound being a D-Lys^6-GnRH-SMPB-pseudomonas exotoxin A chain conjugate.

Fig. 1

A

B

Fig. 2

A   Wh at Hemitoxin

B   Barley Hemitoxin

Fig. 3

1 2 3   4 5   6 7 8   9 10
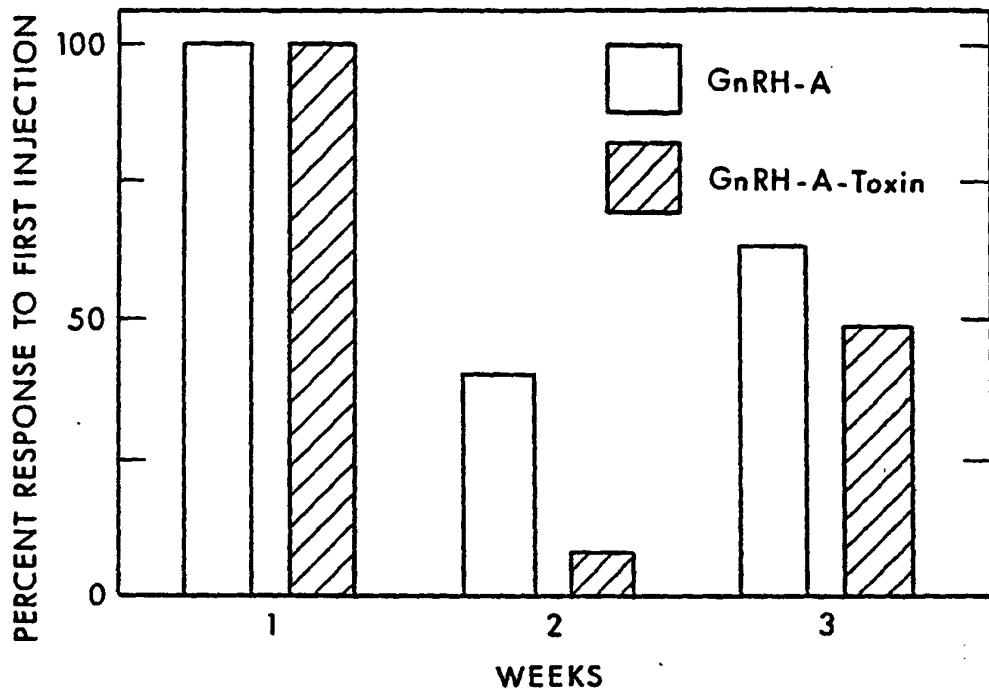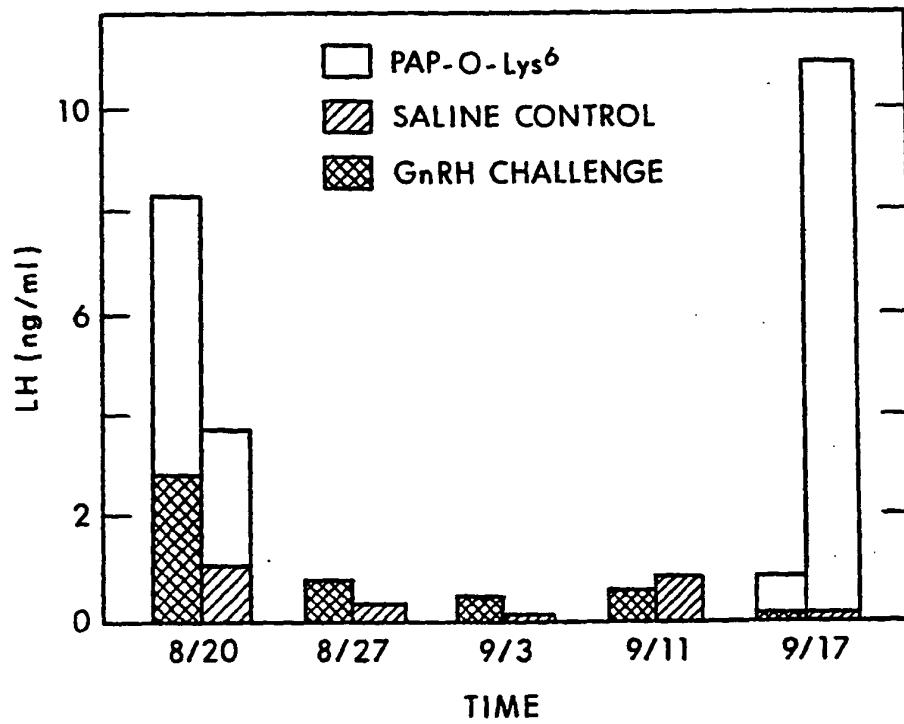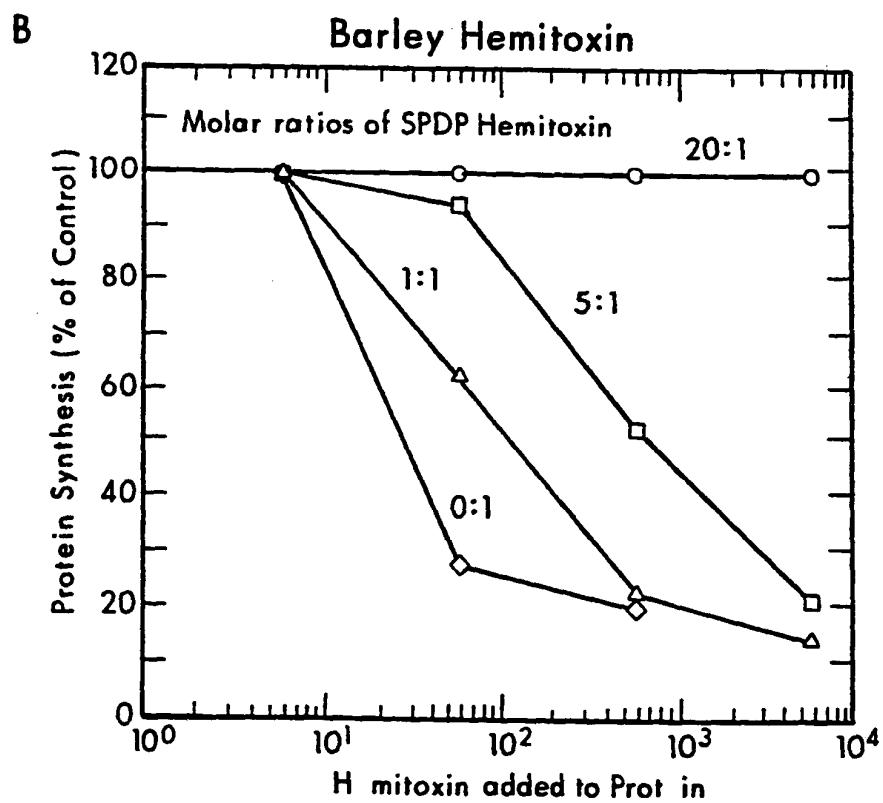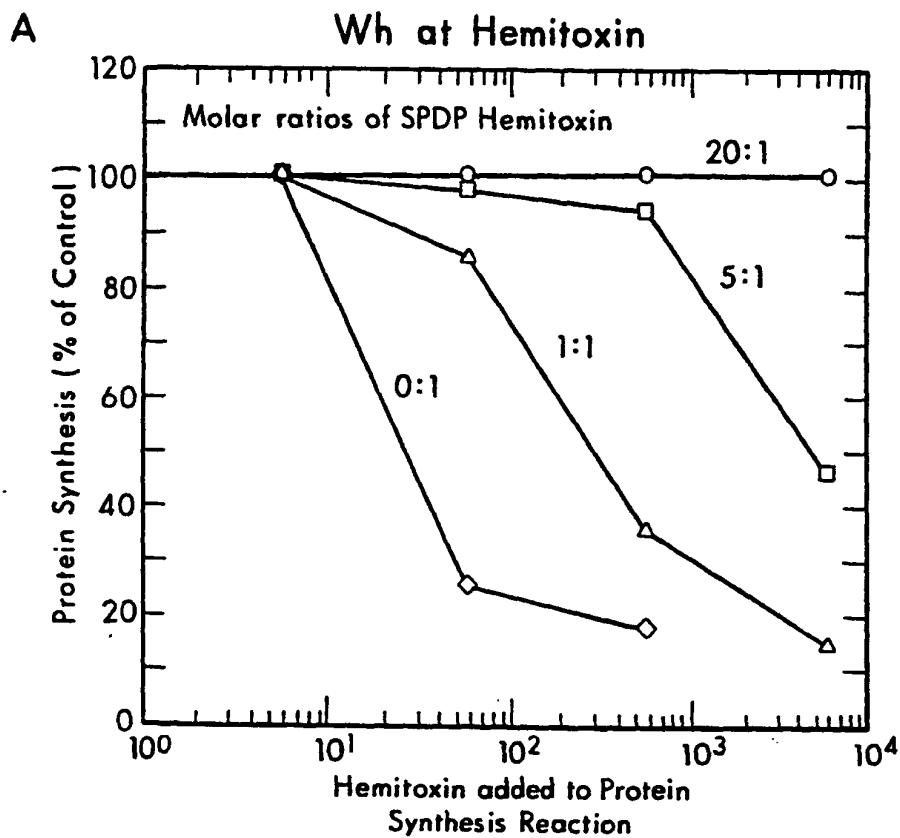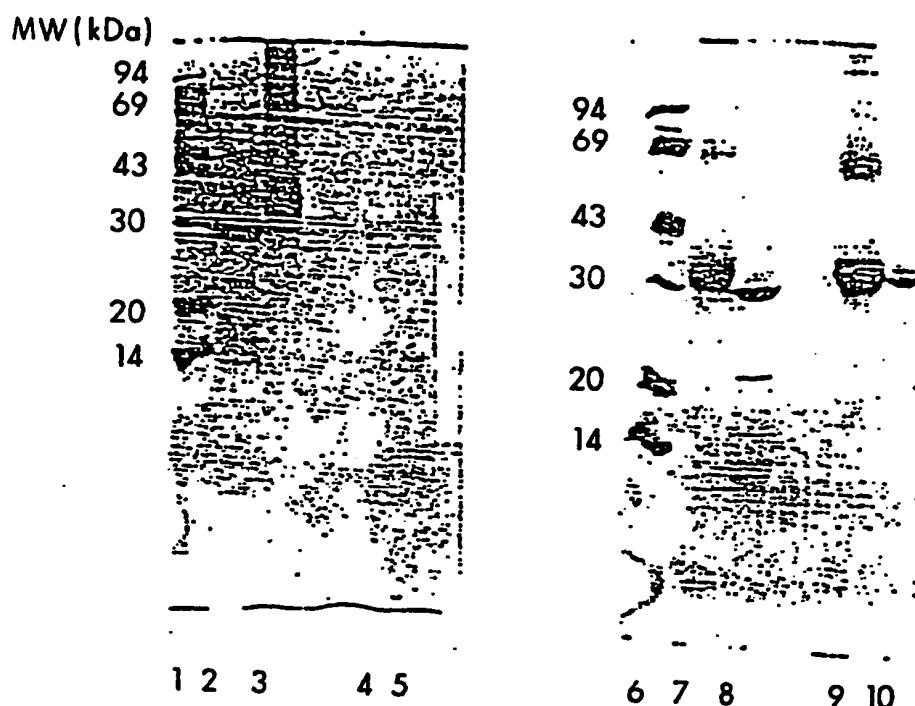
Fig. 4   Effect of 2-Iminothiolane Conjugation
   on Barley Toxin Activity
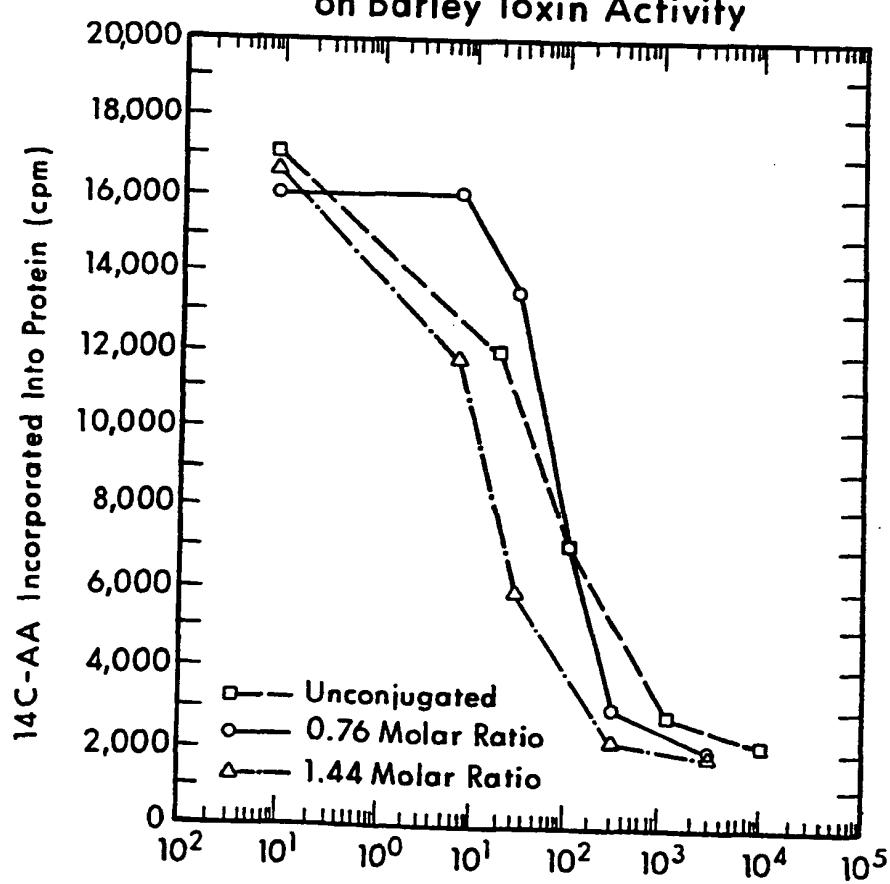
Fig. 4a
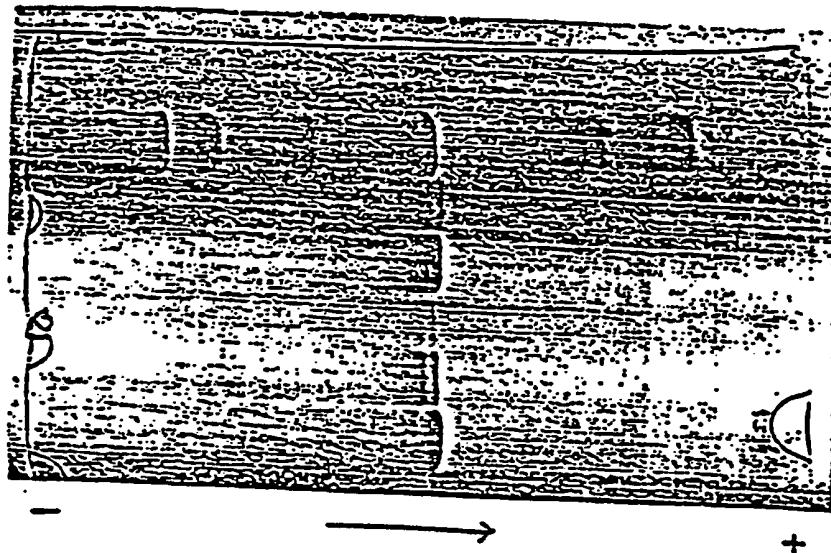
Fig. 5

Toxin Conjugate Receptor Binding
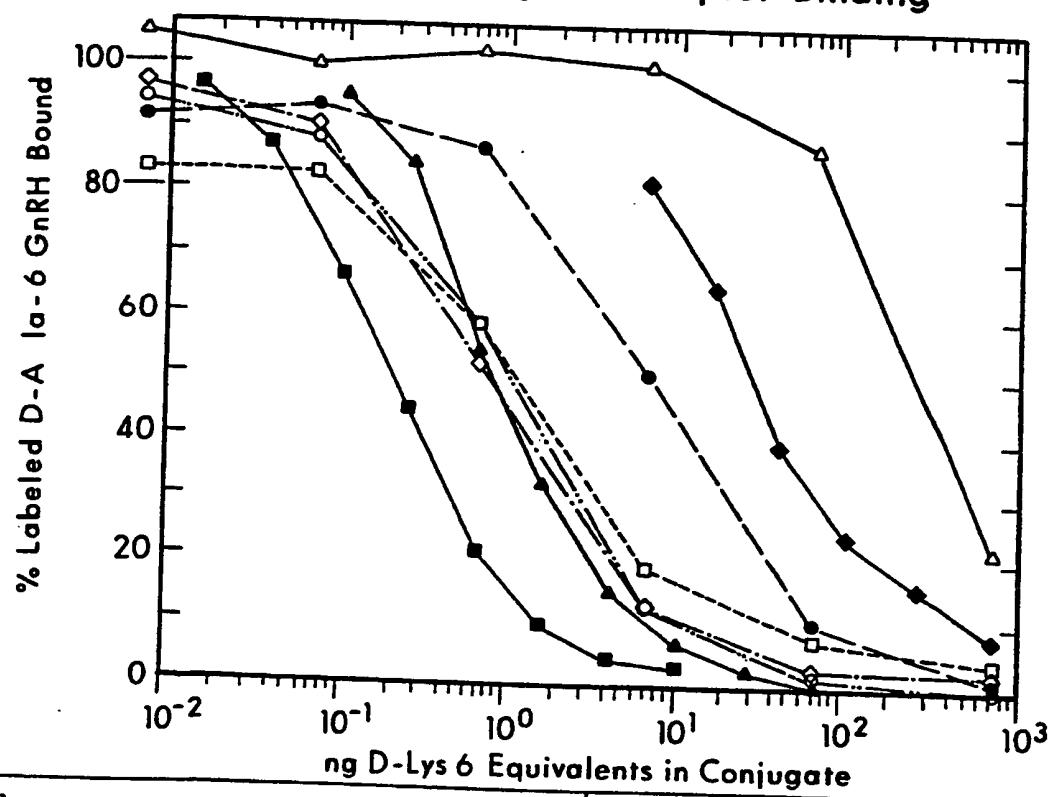
1	—■—	D-Lys-6-GnRH	4	--□--	Geionin-CI-D-Lys-6-GnRH
4.5	—▲—	Barley-2IT-D-Lys-6-GnRH	45	—●—	Barley-CI-D-Lys-6-G
15a	—◆—	PAP-SPDP-D-Lys-6-GnRH	16ac	—△—	Barl y-EACA-CI-D-Lys-6-GnRH
4	--○--	Wheat-CI-D-Lys-6-GnRH			

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/01038

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5) A61K 37/02, 43: C07K 7/06, 08; C07K 17/06
U.S. 514/ 12, 13, 14, 15; 530/324, 325, 326, 327, 409

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
U.S.	514/12, 13, 14, 15 530/324, 325, 326, 327, 409

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	US, A 4,081,533 (Cheesman) 28 MARCH 1978. See abstract.	1-16
A	US, A, 4,201,770 (Stevens) 06 MAY 1980. See abstract.	1-16
A	US, A, 4,302,386 (Stevens) 24 NOVEMBER 1981. See abstract.	1-16
A	US, A, 4,526,716 (Stevens) 02 July 1985. See abstract.	1-16
X Y	US, A 4,691,006 (Stevens) 01 September 1987. See abstract and claims.	1-4,6-7 5,8,9-16
X Y	US, A, 4,767,842 (Stevens) 30 August 1988. See abstract adn claims.	1-4,6-7 5,8,9-16

* Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17 MAY 1990	20 JUL 1990

International Searching Authority	Signature of Authorized Officer
ISA/US	LESTER L. LEE

Form PCT/ISA/210 (second sheet) (Rev.11-87)